APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1988, p. 2027–2030
0099-2240/88/082027-04$02.00/0

Vol. 54, No. 8

Glycyrrhizin Stimulates Growth of *Eubacterium* sp. Strain GLH, a Human Intestinal Anaerobe

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Received 9 February 1988/Accepted 4 May 1988

***Eubacterium* sp. strain GLH was isolated from human feces and produced two kinds of β-D-glucuronidase (EC 3.2.1.31), one new enzyme specific for glycyrrhizin (GL) and the other for phenyl β-D-glucuronides. GL or *p*-nitrophenyl-mono-β-D-glucuronide (pNPG) stimulated the production of GL or pNPG β-glucuronidases and the growth of strain GLH in a basal medium lacking carbohydrate. D-Glucuronic acid also stimulated the growth of the bacterium, but glycyrrhetic acid did not. The increase of GL β-glucuronidase paralleled the growth of the *Eubacterium* strain in pure culture. These results suggest that glucuronides such as GL and pNPG stimulate the growth of the *Eubacterium* strain in a nutrient-poor medium by providing D-glucuronic acid through the activity of β-glucuronidases. The increase in GL β-glucuronidase activity in the presence of GL was observed during the cultivation of human intestinal flora in a general anaerobic medium. During mixed cultivation of the *Eubacterium* strain with *Streptococcus faecalis*, which does not produce GL β-glucuronidase, GL β-glucuronidase was also increased by GL or pNPG, but not by glycyrrhetic acid and *p*-nitrophenol. It is suggested that GL stimulates the growth of strain GLH even in the mixed culture.**

Glycyrrhizin (GL), an active constituent of licorice (which is from *Glycyrrhiza glabra* L., a member of the family *Leguminosae*), is hydrolyzed to the aglycone, 18β-glycyrrhetic acid (GA), and is then converted to 3-epi-18β-glycyrrhetic acid by human intestinal flora, suggesting the presence of 3-dehydro-18β-glycyrrhetic acid as an intermediate (2).

We (1) isolated from human feces a *Eubacterium* sp. capable of hydrolyzing GL to GA and named it *Eubacterium* sp. strain GLH (GL hydrolyzing). This strain produced both GL β-D-glucuronidase, a novel type of β-D-glucuronidase, and a more common type of β-D-glucuronidase (EC 3.2.1.31), which were separated by octyl-Sepharose column chromatography. However, during the course of preliminary experiments, we failed to isolate this bacterium by surveying colonies formed on agar plates because of its slow growth. Strain GLH was obtained only by repeating the transfer of bacterial suspensions capable of hydrolyzing GL in general anaerobic medium (GAM) that contained GL.

In the present paper, we verify that the growth of *Eubacterium* sp. strain GLH is stimulated by GL in a nutrient-poor medium.

MATERIALS AND METHODS

Bacteria and sources. *Eubacterium* sp. strain GLH was isolated in our laboratory from human feces (1) and maintained in GAM semisolid agar. *Streptococcus faecalis* was also isolated from human feces and identified according to *Bergey's Manual* (4). This bacterium did not produce any kinds of β-D-glucuronidase.

Cultivation. A portion of a bacterial suspension of strain GLH precultured in GAM broth was added to GAM broth or to peptone-yeast extract plus Fildes solution (PYF) broth and was then cultured at 37°C in an anaerobic box. PYF broth contains 10 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 5 g of yeast extract, 40 ml of Fildes solution (peptic digest of horse blood), 40 ml of salts solution (0.2 g of $CaCl_2$, 0.2 g of $MgSO_4$, 1 g of K_2HPO_4, 1 g of KH_2PO_4, 10 g of $NaHCO_3$, and 2 g of NaCl in 1 liter) and 0.5 g of L-cysteine HCl in 1 liter, which was used as a basal medium for the growth experiment. In the mixed culture experiment, a portion of each bacterial suspension of strain GLH and of *S. faecalis*, precultured separately in GAM broth, was added simultaneously to GAM broth and then cultivated under anaerobic conditions at 37°C.

Counting of viable cells. Media incubated after inoculation of strain GLH alone were diluted with GAM broth in a 10-fold series, and then aliquots of these diluents were inoculated on GAM agar. After strain GLH was incubated at 37°C for 5 days in an anaerobic box, the number of colonies formed on agar was counted. In the case of a mixed culture of strain GLH and *S. faecalis*, the same procedure was performed. Although the colony shapes of both strains of bacteria were clearly distinguishable, the colonies of the *Eubacterium* strain were confirmed by lack of growth on GAM agar under aerobic conditions.

Thin-layer chromatography. Thin-layer chromatography for GL and GA was performed on silica gel plates (silica gel 60 F-254; layer thickness, 0.25 mm; Merck & Co., Inc., Rahway, N.J.) with the solvent system of acetic acid–*n*-butanol–1,2-dichloroethane–H_2O (4:1:4:1, vol/vol) and of chloroform-petroleum ether-acetic acid (5:5:1, vol/vol), respectively. GL and GA were detected on thin-layer chromatography plates under UV light. The quantities were analyzed with a Shimadzu CS-910 densitometer (λ_s = 250 nm; λ_r = 400 nm), with calibration lines obtained with the authentic samples.

Enzyme assay. Bacteria were harvested and washed once with ethanol to remove GL, GA, and other additives. The cells were suspended in 20 mM potassium phosphate buffer (pH 7.2) or water and were disrupted with a sonicator (model W-220F; Heat Systems-Ultrasonics Inc.) three times for 1 min each time in an ice bath, and then the sonicated cell suspensions were used for the measurement of enzyme activities. Sonication was necessary to obtain full measure-

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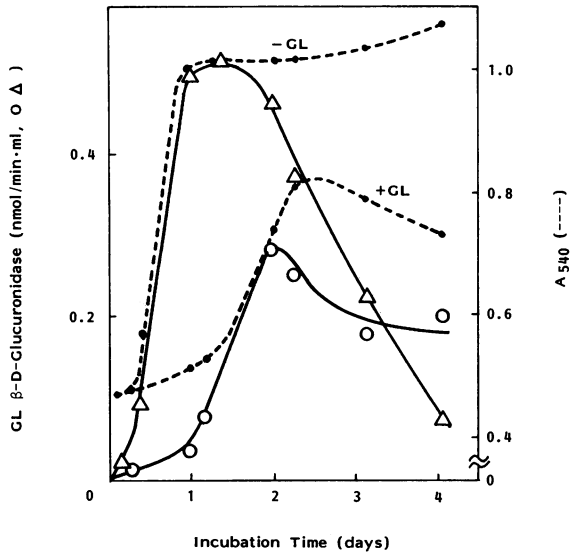

FIG. 1. Growth and GL-hydrolyzing activity of strain GLH cultured in GAM broth with (○) or without (△) 1.2 mM GL. Cell growth was monitored by measuring the A_{540}.

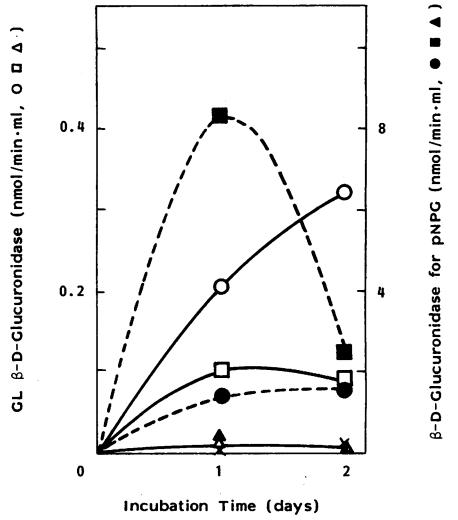

FIG. 2. Effects of 1.2 mM GL (○ and ●), 2.4 mM pNPG (□ and ■), and 14 mM D-glucuronic acid (△ and ▲) on the production of GL- and pNPG-hydrolyzing activities of strain GLH cultured in PYF broth. As a control, nothing was added (×).

ments of activities because native cells showed few β-D-glucuronidase activities.

The enzyme activities for hydrolysis of GL, *p*-nitrophenyl-mono-β-D-glucuronide (pNPG), and phenolphthalein mono-β-D-glucuronide were measured as described previously (1).

Reagents. GL monoammonium and GA were purchased from Tokyo Kasei Kogyo Co., Tokyo, Japan; GL dipotassium salt was a product of Maruzen Kasei Co., Tokyo, Japan; and pNPG was a product of Nakarai Chemicals, Ltd., Kyoto, Japan. Phenolphthalein mono-β-D-glucuronide was purchased from Sigma Chemical Co., St. Louis, Mo. GAM was a product of Nissui Seiyaku Co., Tokyo, Japan. All other reagents were of the best available commercial quality.

RESULTS

Effects of GL on growth and GL β-glucuronidase activity of strain GLH. *Eubacterium* sp. strain GLH grown in GAM broth showed GL-hydrolyzing activity that paralleled its slow growth (1). The addition of GL to GAM caused both the bacterial growth and the GL-hydrolyzing activity to decrease similarly (Fig. 1), although GL was hydrolyzed to GA, which was not further metabolized. These results suggest that GL and GA have weak antibacterial action. In fact, GL and GA inhibited the colony formation of strain GLH on GAM agar.

Stimulation of GL-hydrolyzing activity and growth of *Eubacterium* sp. strain GLH by GL. Although GL decreased the GL-hydrolyzing activity of strain GLH in GAM broth containing 0.3% glucose, GL increased remarkably the activity during cultivation in the basal medium (PYF broth) containing no carbohydrate (Fig. 2). GL-hydrolyzing activity was also increased by the addition of pNPG into PYF medium, but not by the addition of D-glucuronic acid or of GA. Moreover, pNPG-hydrolyzing activity was markedly increased by the addition of pNPG but only slightly increased by the addition of GL (Fig. 2).

The growth of strain GLH in PYF medium was also stimulated by the addition of GL (Table 1), despite the inhibition of the bacterial growth by GL in GAM broth (Fig. 1). pNPG or D-glucuronic acid also stimulated the growth of the bacterium in PYF medium, but GA did not. These results indicate that glucuronide compounds such as GL or pNPG and D-glucuronic acid stimulate the growth of strain GLH in a nutrient-poor medium. Moreover, the values of GL-hydrolyzing activities per cell (1.5×10^{-10}, 1.9×10^{-12}, or 2.3×10^{-12} μmol/min per cell in the presence of GL, pNPG, or D-glucuronic acid, respectively) were calculated from Fig. 2 and Table 1. Enzyme activity per cell corresponds to the specific activity; therefore, it is suggested that GL β-glucuronidase is induced by GL.

Effects of GL on human intestinal flora. When fresh human feces were diluted and then inoculated into GAM broth, only very low GL-hydrolyzing activities were observed during 4 days of cultivation. The addition of GL markedly increased GL- and pNPG-hydrolyzing activities (Fig. 3), though a considerable amount of the latter activity was observed without the addition of GL.

Effects of GL on a mixed culture of *Eubacterium* sp. strain GLH and *S. faecalis*. Because it was difficult to count the viable cell number of strain GLH during cultivation of human feces, the effects of GL on a mixed culture of *Eubacterium* sp. and *S. faecalis*, which produces no β-glucuronidase and forms a colony shape different from that of *Eubacterium* sp., were studied. When two strains of these bacteria were cultivated in GAM broth, only very low GL- and pNPG-hydrolyzing activities were observed during 4 days of cultivation. This phenomenon is similar to that

TABLE 1. Stimulation of growth of *Eubacterium* sp. strain GLH by GL and pNPG in PYF broth

Addition (concn)	No. of viable cells/ml of culture medium[a]
Nothing	2.7×10^5
GL (1.2 mM)	1.3×10^6
pNPG (2.4 mM)	5.2×10^7
D-Glucuronic acid (14 mM)	4.4×10^6

[a] After 24 h of cultivation.

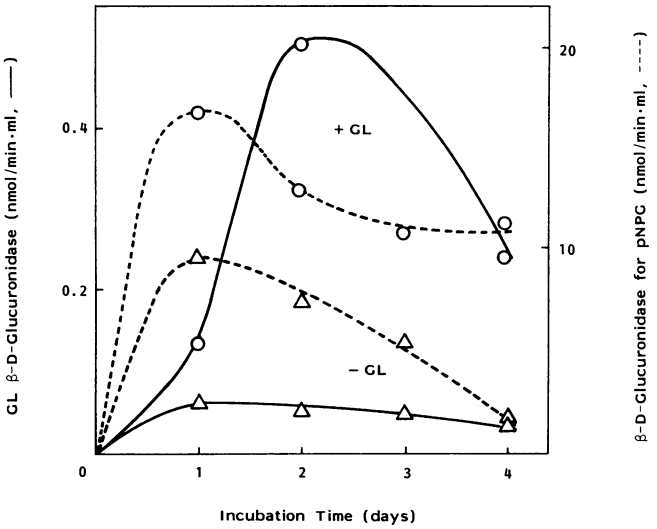

FIG. 3. Effects of GL on GL-hydrolyzing activities of human intestinal flora. Human feces obtained were immediately diluted in a 10-fold series, and then a portion (0.1 ml) of 10^3-fold diluent was inoculated into 100 ml of GAM broth with (○) or without (△) 1.2 mM GL.

observed when GAM medium was incubated with human feces (Fig. 3). However, GL-hydrolyzing activity was increased by the addition of GL to the overnight mixed culture and was similarly increased by the addition of pNPG (Fig. 4). Furthermore, pNPG-hydrolyzing activity was remarkably increased by pNPG and slightly increased by GL (data not shown). On the other hand, D-glucuronic acid was not effective in stimulating the activities of these enzymes, and GA and *p*-nitrophenol at the same concentrations as their β-glucuronides decreased these activities. When GL was added to a mixed culture 24 h after cultivation, the *Eubacterium* growth paralleled the increase of GL-hydrolyzing activity, but the growth of *S. faecalis* was not affected (Fig. 5). pNPG also stimulated the growth of strain GLH and the enzyme activity in the mixed culture. These results indicate that glucuronides stimulate the growth of strain GLH in the mixed culture as they stimulate growth of a single culture in a nutrient-poor medium. In the case of the mixed culture in GAM broth, *S. faecalis* grows by consuming glucose until the medium becomes deficient in nutrients, after which strain GLH starts to grow and to produce GL-hydrolyzing activity.

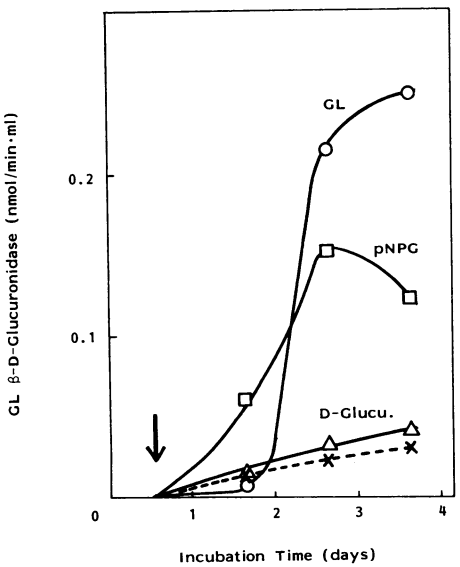

FIG. 4. Effects of 1.2 mM GL, 2.4 mM pNPG, and 2.4 mM D-glucuronic acid on GL-hydrolyzing activities in the mixed culture of strain GLH and *S. faecalis* in GAM broth. Additives were added to overnight culture as indicated by the arrow. As a control, nothing was added to the mixed culture (×).

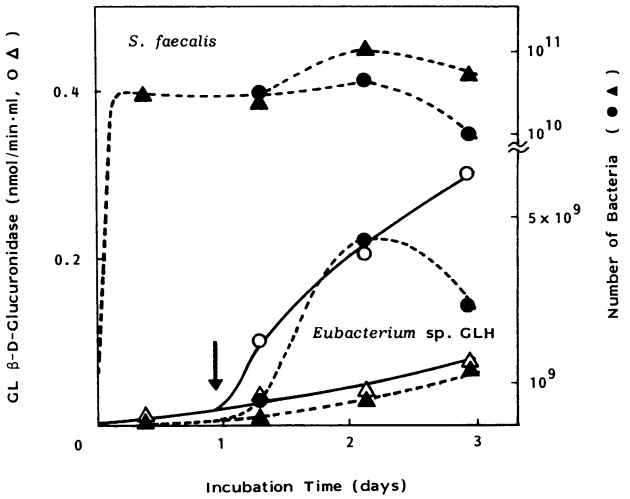

FIG. 5. Effects of GL on growth (– – – –) and GL β-D-glucuronidase activity (——) of strain GLH and *S. faecalis*, with 1.2 mM GL (○ and ●) or nothing (△ and ▲) added 24 h after cultivation (arrow).

DISCUSSION

Eubacterium sp. strain GLH produced two kinds of β-glucuronidase: GL β-glucuronidase and usual β-glucuronidase (EC 3.2.1.31) (1). GL stimulated the growth and GL-hydrolyzing activity of the *Eubacterium* sp. in a nutrient-poor medium containing no carbohydrate (Table 1 and Fig. 2), despite the inhibition of growth and the decrease of GL-hydrolyzing activity of the bacterium by GL in GAM broth (Fig. 1). pNPG and D-glucuronic acid also stimulated the growth of strain GLH in a nutrient-poor medium, but GA, the aglycone of GL, did not. Moreover, D-glucuronic acid was released from GL and pNPG by GL- and usual β-glucuronidases, respectively (1), and GA was not metabolized by the bacterium at all. Accordingly, the growth of strain GLH in a nutrient-poor medium seems to be stimulated by D-glucuronic acid liberated from GL and pNPG by the corresponding β-glucuronidases. Although 14 mM D-glucuronic acid stimulated the proliferation of the *Eubacterium* strain, the bacteria fermented only a small amount of D-glucuronic acid, judging from the weak acidity (pH 6.7) of the medium after cultivation; moreover, the stimulatory effects of D-glucuronic acid were far less than those of 14 mM glucose or fructose, on which the growth of the bacteria exceeded 10^9 cells per ml and the pH of the medium decreased to between 5.0 and 5.5. It is possible that strain GLH transports the glucuronide compounds more efficiently than glucuronic acid, since 1.2 mM GL or 2.4 mM pNPG stimulated the growth of the bacterium to an extent similar to that caused by 14 mM D-glucuronic acid. On the basis of the

values of GL-hydrolyzing activities per cell in the presence of GL, pNPG, or D-glucuronic acid in PYF medium, calculated from Fig. 2 and Table 1, we suggest that GL induces the synthesis of GL β-glucuronidase and, in turn, allows *Eubacterium* sp. strain GLH to grow.

Eubacterium sp. strain GLH grew very slowly even in GAM broth (Fig. 1), but *S. faecalis* grew rapidly. When the two bacterial strains were inoculated simultaneously in GAM broth, *S. faecalis* grew rapidly and consumed glucose, thus preventing the growth of strain GLH. However, since *S. faecalis* does not produce any kinds of β-glucuronidase, only the growth of strain GLH was stimulated by the addition of glucuronides such as GL and pNPG. In GAM broth inoculated with human feces and in lower parts of the human intestine, rapidly growing bacteria grow first and consume nutrients. Therefore, slowly growing bacteria such as *Eubacterium* sp. strain GLH grow under nutrient-poor conditions. However, the possession of a unique enzyme, GL β-glucuronidase, which is not produced by other intestinal bacteria (1, 3), seems to allow strain GLH to grow on the glucuronic acid liberated from GL, a compound that cannot be metabolized by other bacteria, as is suggested in Fig. 3. Accordingly, when GL is administered orally to humans as a sweetener or as licorice (an oriental medicine, which contains GL as an active constituent) GL β-glucuronidase and the enzyme-producing bacteria seem to increase in the intestine. Nakano et al. (5) reported that GL was not detected in the sera of human subjects after oral administration of GL; only GA was detected. However, with intravenous injection, GL and GA were detected in the sera, as determined by enzyme immunoassay. Moreover, rat liver homogenates did not show GL-hydrolyzing activity (unpublished data). These results suggest that bacteria such as *Eubacterium* sp. which produce GL β-glucuronidase contribute greatly to hydrolysis of GL to GA in humans administered GL orally.

ACKNOWLEDGMENT

We thank S. Takayanagi for secretarial assistance.

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